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Evidence That Protein Kinase C (PKC) Participates in the Meiosis I to Meiosis II Transition in Mouse Oocytes

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Oocytes from LTXBO mice exhibit a delayed entry into anaphase I and frequently enter interphase after the first meiotic division. This unique oocyte model was used to test the hypothesis that protein kinase C (PKC) may regulate the meiosis I-to-meiosis II transition. PKC activity was detected in LTXBO oocytes at prophase I and increased with meiotic maturation, with the highest ($P < 0.05$) activity observed at late metaphase I (MI). Treatment of late MI-stage oocytes with the PKC inhibitor, bisindolylmaleimide I (BIM), transiently reduced ($P < 0.05$) M-phase-promoting factor (MPF) activity and promoted ($P < 0.05$) progression to metaphase II (MII), while mitogen-activated protein kinase (MAPK) activity remained elevated during the MI-to-MII transition. Confocal microscopy analysis of LTXBO oocytes during this transition showed PKC-δ associated with the meiotic spindle and then with the chromosomes at MII. Inhibition of PKC activity also prevented untimely entry into interphase, but only when PKC activity was reduced in oocytes before the progression to MII and thus indicates that the transition into interphase is directly associated with the delayed triggering of anaphase I. Moreover, the defect(s) that initiate activation occur upstream of MAPK, as suppression of PKC activity failed to prevent activation by *Mos*^{tm1Ev}/ *Mos*^{tm1Ev} LTXBO oocytes expressing no detectable MAPK activity. In summary, PKC participates in the regulatory mechanisms that delay entry into anaphase I in LTXBO oocytes, and the disruption promotes untimely entry into interphase. Thus, loss of regulatory control over PKC activity during oocyte maturation disrupts the critical MI-to-MII transition, leading to a precocious exit from meiosis.

Key Words: **meiosis; anaphase I; protein kinase C; MPF; spontaneous activation; PKC-δ; MAPK.**

INTRODUCTION

Upon release from prophase I, a fully grown oocyte must undergo two consecutive M phases without an intermediate S phase to complete meiosis successfully. Similar to the mitotic cell cycle, meiosis is regulated by oscillations in the activity of M-phase-promoting factor (MPF), a heterodimer composed of a catalytic p34^{cdc2} kinase and a cyclin B-regulatory subunit (Norbury and Nurse, 1992; Murray, 1995). Entry into metaphase is driven by activation of MPF, while entry into anaphase is correlated with ubiquitin-mediated cyclin B degradation and a decrease in MPF activity (Choi *et al.*, 1991; Verlhac *et al.*, 1994; Hampl and Eppig, 1995; Townsley and Ruderman, 1998). The two successive M phases in meiosis are distinct, and suggest that MPF might be subject to different control mechanisms during metaphase I (MI) and metaphase II (MII). Meiosis I is unique in that it is reductional and normally proceeds directly into a second metaphase. The mechanisms that regulate the transition from MI to MII must allow for the separation of homologous chromosomes, while maintaining cohesion between sister chromatids and preventing entry into interphase. During this transition, cyclin B is degraded and MPF activity decreases transiently, but is then restabilized during MII (Kubiak *et al.*, 1992; Verlhac *et al.*, 1994; Hampl and Eppig, 1995; Polanski *et al.*, 1998). Oocytes normally remain arrested at MII with high MPF activity. Cytostatic factor (CSF) is described as the activity in vertebrate eggs that restrains the cell cycle in MII (Masui, 1991; Kubiak *et al.*, 1993; Verlhac *et al.*, 1994); however, the role and regulation of CSF during MI, if any, remain unclear. The product of the *Mos* proto-oncogene (MOS), its target, mitogen-activated protein kinase kinase (MAPKK/

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MEK1), and the MEK1 substrate MAPK are essential components of CSF (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1996; Sagata *et al.*, 1997). The completion of meiosis is initiated by fertilization or parthenogenetic activation, which triggers anaphase II. The second meiotic division is similar to mitosis, as MPF is inactivated coincident with the separation of sister chromatids and entry into interphase (Murray, 1995; Townsley and Ruderman, 1998).

Although progression from MI to MII is a unique and critical transition during meiosis, the regulatory factors that control the first meiotic division remain inadequately understood. One possible regulator of meiosis I is protein kinase C (PKC). Mounting evidence indicates that PKC can function as a key regulator of critical cell-cycle transitions during mitosis, including the G_1/S and G_2/M, in different cell types (Livneh and Fishman, 1997; Black, 2000). PKC-mediated regulation of these transitions may be either negative or positive, depending on the timing of PKC activation during the cell cycle and the specific PKC isoforms involved (Livneh and Fishman, 1997; Black, 2000). The PKC family consists of 11 different serine/threonine kinases that are generally subdivided into three groups based on sequence homology, as well as activator and cofactor requirements. These groups include the "conventional" or "classical" (PKC-α, -βI, -βII, and -γ), "novel" (PKC-δ, -ϵ, -θ, -μ and -η), and "atypical" (PKC-λ, and -ζ) isoforms (Mellor and Parker, 1998). PKC is expressed in mouse oocytes and can influence the progression of meiosis. Expression of PKC-α, -β, -δ, -λ and -ζ proteins has been detected in oocytes arrested at prophase I and in mature MII eggs; however, only mRNA for PKC-δ and -λ are detected (Gangeswaran and Jones, 1997; Luria *et al.*, 2000; Pauken and Capco, 2000; Downs *et al.*, 2001). Direct activation of PKC in oocytes at prophase I with an intact germinal vesicle (GV) inhibits spontaneous resumption of meiosis (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986; Lefevre *et al.*, 1992; Downs *et al.*, 2001). A role for PKC in the transition into the first mitotic cycle upon egg activation has also been proposed, but remains unresolved. Whereas some studies suggest that PKC activation promotes entry into interphase by *Xenopus* and mouse MII eggs (Bement and Capco, 1991; Colonna *et al.*, 1997; Gallicano *et al.*, 1997a,b), others indicate that PKC does not induce complete egg activation and cell-cycle resumption (Moore *et al.*, 1995; Ducibella and Lefevre, 1997). Given that PKC can influence the key transitions that demarcate the resumption of and possible exit from meiosis, it may also regulate the transition from MI to MII. This hypothesis is supported by earlier studies that demonstrate PKC activation in mouse oocytes, after the resumption of meiosis, blocks progression to MII (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986).

Oocytes from strain LTXBO mice provide a unique model to test the hypothesis that PKC plays a role in the regulation of meiosis I. These oocytes, and those from related mouse strains LT/Sv and LTXBJ (hereafter referred to as LT oocytes) exhibit defects in the regulatory mechanisms that govern the first meiotic division (Stevens and Varnum, 1974; Eppig *et al.*, 1977, 1996). Similar to normal oocytes, fully grown LT oocytes, resume meiosis when released from the follicular environment and form the first meiotic spindle (Ciemerych and Kubiak, 1998). However, these oocytes show defects in the subsequent progression of meiotic maturation and remain at MI for an extended period (Eppig *et al.*, 1996), with a high proportion ovulated as primary oocytes at MI (Kaufman and Howlett, 1986; O'Neill and Kaufman, 1987). The prolonged MI is due to a delayed triggering of anaphase I (Ciemerych and Kubiak, 1998; Hirao and Eppig, 1999) and is maintained by continued high MPF activity that is sustained, at least in part, by restricted degradation of cyclin B (Hampl and Eppig, 1995). Most late MI-stage LT oocytes eventually enter anaphase I and extrude the first polar body; however, a significant percentage of these eggs enter interphase after the first meiotic division, with no apparent arrest at MII, and cleave the 2-cell stage (Maleszewski and Yanagimachi, 1995; Eppig *et al.*, 1996). This premature transition into the first mitosis in oocytes retained within the ovary leads to blastocyst development and potential ovarian teratoma formation (Stevens and Varnum, 1974; Eppig *et al.*, 1977, 1996). Defects in the regulatory mechanisms that govern the progression of meiosis I and promote spontaneous activation are intrinsic to LT oocytes (Eppig *et al.*, 2000). Hence, a link between the meiotic defects in LT oocytes and aberrant PKC activity and/or regulation would support a role for PKC during the normal progression of meiosis I. This study was therefore undertaken to evaluate PKC activity during meiotic maturation and to determine whether modulation of PKC activity influences the triggering of anaphase I, and subsequent entry into interphase by LT oocytes.

METHODS

Mice

All mice were bred and raised in the research colony of the authors at the Jackson Laboratory. Prepubertal, 20- to 23-day-old LTXBO mice were used for all experiments. LTXBO is a recombinant inbred strain derived from LT/Sv and C57BL/6-J produced by L. C. Stevens and D. S. Varnum at the Jackson Laboratory (unpublished observations). Oocytes from LTXBO mice exhibit a prolonged MI-stage and a higher incidence of spontaneous parthenogenetic activation than its LT/Sv progenitor (Eppig *et al.*, 1996). Prepubertal LTXBO mice homozygous for a null allele of *Mos*, *Mos*^{tm1Ev}, were also used for some experiments (Colledge *et al.*, 1994; Hirao and Eppig, 1997). These mice were genotyped by polymerase chain reaction (PCR) using specific *Mos* and neo primer sets as previously described (Hirao and Eppig, 1997).

Oocyte Isolation and Culture Conditions

Mice were injected with 5 IU equine chorionic gonadotrophin (eCG) to stimulate preovulatory follicle development, and

cumulus-enclosed oocyte complexes (COC) were isolated 44–48 h later, as previously described (Eppig *et al.,* 1996). GV-stage oocytes were obtained by denuding the oocytes immediately after recovery from the ovary. For *in vitro* oocyte maturation, COC were cultured in 10 × 35-mm petri dishes (Falcon 1008) for various specified times, depending on the experiment, in 3 ml Minimal Essential Medium (MEM) supplemented with 3 mg/ml crystallized bovine serum albumin (BSA; Life Technologies, Rockville, MD). All cultures were maintained at 37°C in a modular incubation chamber (Billups-Rothenberg, Del Mar, CA) equilibrated with 5% CO_2, 5% O_2, and 90% N_2. At the end of a specified culture period, the oocytes were denuded, by repeatedly drawing the complexes in and out of a small bore pipette, then examined using a stereomicroscope to determine the progression of meiotic maturation. Emission of the first polar body was used as an indication of progression to MII. Oocytes that had resumed meiosis and undergone germinal vesicle breakdown (GVBD), but had not extruded a polar body after a 14-h culture period were classified as late MI-stage oocytes. For various experiments, the subsequent culture of denuded oocytes was undertaken in stoppered borosilicate glass tubes containing 1 ml of medium under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in a 37°C water bath.

PKC Activity

Phosphorylation of a specific PKC substrate, myristoylated alanine-rich C kinase substrate or MARCKS peptide (Blackshear, 1993; Gallicano *et al.,* 1997), was used to measure PKC activity in LTXBO oocytes at different stages of meiotic maturation. Oocytes were evaluated at the GV-stage, as well as at 4, 8, 14, and 18 h after cumulus-enclosed culture in MEM. At the end of culture, the cumulus cells were removed and denuded oocytes were frozen for subsequent analysis. To evaluate the efficacy of a specific PKC inhibitor, bisindolylmaleimide I (BIM; Calbiochem, San Diego, CA) in reducing endogenous PKC activity during culture, late MI-stage oocytes were denuded and incubated in MEM supplemented with 1.0 μM BIM for 4 h. Original stock solutions of BIM were prepared with DMSO, while subsequent working concentrations were prepared with culture medium. Only 1 μl of the BIM solution in DMSO was added per 1 ml of medium (0.1%); an equal volume of DMSO alone was added to the control group. For each reaction, groups of 20 denuded oocytes were collected (after culture) and washed in homogenization buffer (HB) containing 80 mM sodium β-glycerophosphate (pH 7.5), 15 mM $MgCl_2$, 20 mM EGTA, 0.5 mM EDTA, 2 mM Na_3VO_4, 1 mg/ml BSA, 1 mM DTT, 1 mM pefabloc, as well as 10 μg/ml of leupeptin, aprotinin, and pepstatin. The oocytes were frozen in 10 μl of HB buffer at −80°C until use. At the time of assay, 12 μl of the reaction buffer containing 10 μM synthetic inhibitor of cAMP-dependent protein kinase A (Sigma Chemical, St. Louis, MO), 5 mg/ml MARCKS phosphorylated site domain (psd) peptide (BIOMOL, Plymouth Meeting, PA), and 2.5 μCi/ml [γ-^{32}P]ATP (New England Nuclear Research Products, Boston, MA) were added to each sample. Negative control samples of GV-stage oocytes were evaluated in which 1.0 μM BIM was added to the reaction buffer or the MARCKS substrate was omitted. The reaction was allowed to proceed for 45 min at 30°C, then stopped by the addition of an equal volume of twice-concentrated sample buffer (Laemmli, 1970). The samples were heated to 100°C for 5 min then subjected to SDS–PAGE in an 18% polyacrylamide gel. The gel was dried and the incorporation of the radioactive isotope was visualized and quantified by using a phosphorimager (Fuji Bio-Imaging Analysis System; Fuji Medical Systems USA, Stanford, CT).

Modulation of PKC Activity in Late MI-Stage Oocytes

The effect of PKC activity in late MI-stage LTXBO oocytes on the progression to MII was determined. In preliminary experiments, timing of emission of the first polar body was evaluated after varying COC culture periods; it was found that some LTXBO oocytes begin to undergo a late MI-to-MII transition between 14 and 16 h after *in vitro* maturation. LTXBO oocytes were therefore matured cumulus-enclosed for 14 h and denuded; oocytes that had not extruded a first polar body (late MI-stage) were placed in culture for an additional 1 or 2 h in MEM or MEM supplemented with 1.0 μM BIM, a specific PKC inhibitor. At the end of culture, the oocytes were evaluated by using a stereomicroscope to determine the incidence of progression to MII, as indicated by emission of the first polar body. The oocytes were then fixed and stained with propidium iodide to evaluate chromatin configuration.

The effect of PKC activity on spontaneous activation by LTXBO oocytes was also evaluated. To determine the response to a decrease in PKC activity, late MI-stage oocytes were collected after a 14-h cumulus-enclosed culture, denuded, and incubated in MEM supplemented with increasing concentrations (0, 0.1, 0.5, and 1.0 μM) of BIM for an additional 10 h. In a separate experiment to determine the influence of PKC stimulation on oocyte activation, denuded late MI-stage oocytes were treated with increasing concentrations (0, 0.1, 1.0, and 5.0 nM) of the biologically active phorbol ester phorbol-12-myristate-13-acetate (PMA; Calbiochem), a PKC agonist. The oocytes were treated with PMA for 30 min in MEM, then washed thoroughly (six times) and transferred to fresh media for an additional 10-h culture. At the end of this period (approximately 24 h after initial COC recovery), the oocytes were washed, transferred to fresh MEM, and examined for progression to MII as well as pronuclear (PN) formation to indicate entry into interphase. The oocytes were cultured for an additional 18 h (until 42 h after initial COC recovery) to evaluate completion of the first mitotic cycle as evident by development to the two-cell stage.

To determine when oocytes were most responsive to modulation of PKC activity, late MI-stage oocytes were collected and denuded after a 14-h cumulus-enclosed culture. These oocytes were treated with 1.0 μM BIM either immediately (at 14 h) or at subsequent 2-h intervals until 20 h (16, 18, and 20 h after initial COC recovery). Spontaneous PN formation and development to the two-cell stage were evaluated at 24 and 42 h after initial COC recovery, respectively.

A final experiment was undertaken to determine whether suppression of PKC activity affects spontaneous activation by oocytes from LTXBO mice bearing a null allele for *Mos*. Previous studies confirmed no detectable MAPK activity in oocytes from Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice (Hirao and Eppig, 1997). Late MI-stage oocytes from Mos^{tm1Ev}/Mos^{tm1Ev} and $Mos^{tm1Ev}/^{+}$ LTXBO mice were collected and cultured in MEM or MEM supplemented with 1.0 μM BIM. The oocytes were subsequently evaluated to assess progression to MII and PN formation, as previously described.

MPF and MAPK Activity

Both MPF and MAPK activity were measured in LTXBO oocytes during the MI to MII transition, in response to BIM treatment. After a 14-h cumulus-enclosed culture, LTXBO oocytes were

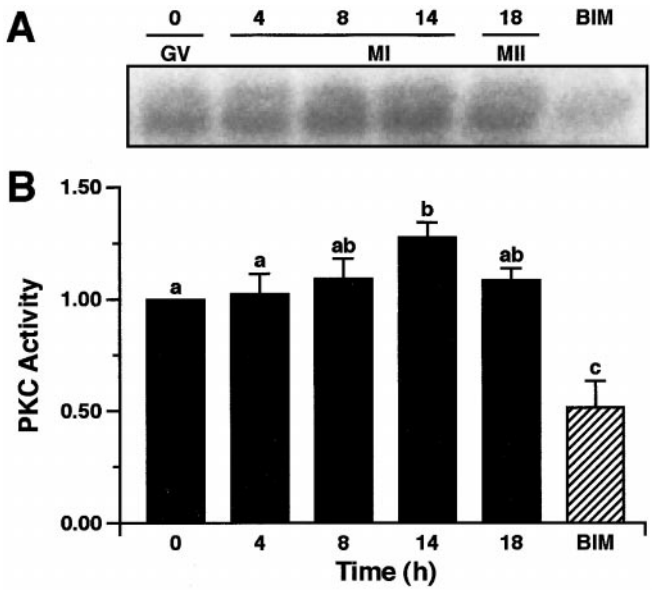

FIG. 1. PKC activity is elevated in late MI-stage LTXBO oocytes. Phosphorylation of MARCKS psd peptide was used to measure PKC activity in LTXBO oocytes during meiotic maturation. Oocytes were collected at the GV-stage, as well as at 4, 8, 14, and 18 h post cumulus-enclosed culture. A representative gel (A) is shown, in which a total of 20 denuded oocytes were used per reaction. Densitometry analysis was performed and all data are expressed as a percent (mean ± SEM) of the value at the GV-stage (B). PKC activity was detected at the GV-stage and increased during meiotic maturation, with the highest activity observed in late MI-stage oocytes collected after a 14-h culture period. PKC activity was reduced when denuded late MI-stage oocytes were treated with a PKC inhibitor (1.0 μM BIM) for 4 h. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

denuded and cultured in MEM or MEM supplemented with 1.0 μM BIM. Oocytes from the control and BIM-treated groups were collected at 30-min intervals for the next 2 h (0, 0.5, 1, 1.5, and 2 h) and 4 h after initial BIM treatment. MPF and MAPK activities were measured in the same sample by assessing the phosphorylation of histone H1 and myelin basic protein (MBP), respectively (Kubiak *et al.*, 1993; Hirao and Eppig, 1997). For each reaction, groups of 10 oocytes were collected and washed in homogenization buffer (HB) containing 15 mM $MgCl_2$, 80 mM sodium β-glycerophosphate (pH 7.5), 20 mM EGTA, 0.5 mM EDTA, 2 mM Na_3VO_4, 1 mg/ml BSA, 1 mM DTT, 1 mM pefabloc, as well as 10 μg/ml of leupeptin, aprotinin, and pepstatin. The oocytes were frozen in 10 μl of HB buffer at −80°C until use. At the time of assay, 15 μl of the reaction buffer containing 10 μM synthetic inhibitor of cAMP-dependent protein kinase A (Sigma), 1 mg/ml histone H1 (Type III, Sigma), 2.5 mg/ml MBP (Sigma) and 2.5 μCi/ml [γ-^{32}P]ATP (New England Nuclear Research Products) were added to each sample. The reaction was allowed to proceed for 45 min at 30°C, and then stopped by the addition of an equal volume of twice-concentrated sample buffer (Laemmli, 1970). The samples were heated to 100°C for 5 min and then subjected to SDS–PAGE in a 15% polyacrylamide gel. The gel was dried and the incorporation of the radioactive isotope was visualized and quantified with a phosphorimager (Fuji Bio-Imaging Analysis System).

Immunocytochemistry

The subcellular localization pattern of a "novel" isoform of PKC, PKC-δ, was evaluated in LTXBO oocytes at the GV-stage and during the MI-to-MII transition. GV-stage oocytes were obtained by denuding the oocytes immediately after COC recovery from the ovary. To obtain oocytes undergoing the MI-to-MII transition, late MI-stage oocytes were denuded after a 14-h cumulus-enclosed culture and incubated in MEM. Groups of 25 oocytes were collected at 30-min intervals for the next 2 h (0, 0.5, 1, 1.5, and 2 h), and a final group was collected after a 4-h incubation. The oocytes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), supplemented with 1 mg/ml polyvinyl-pyrrolidone (PVP), for 4 h at 4°C. After fixation, the oocytes were transferred to blocking solution (PBS with 10% fetal bovine serum) and stored at 4°C. All steps in the immunostaining procedure were carried out at room temperature and all solutions were supplemented with 0.1% saponin (Sigma). The oocytes were initially placed in blocking solution with saponin for 1 h, then rinsed in wash buffer (PBS with 1 mg/ml PVP) and incubated with 1 μg/ml anti-PKC-δ (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After incubation with the primary antibody, the oocytes were washed and exposed to 3 μg/ml of FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The oocytes were then transferred to propidium iodide (1 μg/ml in PBS) for 10 min, washed, and mounted onto poly-L-lysine-coated slides using vectashield mounting medium (Vector Laboratories, Burlingame, CA). Negative control samples were evaluated in which the primary antibody was omitted. Expression of PKC-δ was assessed by using a TCS-NT laser scanning confocal microscope equipped with an air-cooled argon ion laser system (Leica Microsystems).

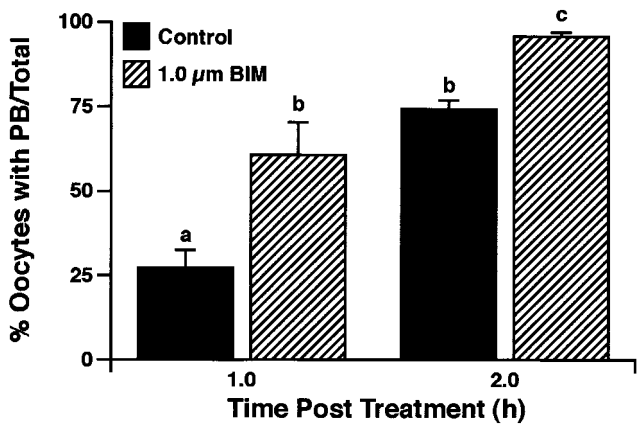

FIG. 2. Treatment with a specific PKC inhibitor (BIM) promotes entry into anaphase I and emission of the first polar body by late MI-stage LTXBO oocytes. COC were collected and placed in culture for 14 h. At the end of culture, the cumulus cells were removed and denuded late MI-stage oocytes were cultured in MEM (solid bars) or MEM supplemented with 1.0 μM BIM (hatched bars). The oocytes were evaluated at 1 and 2 h post treatment to determine the incidence (mean ± SEM) of first polar body emission. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

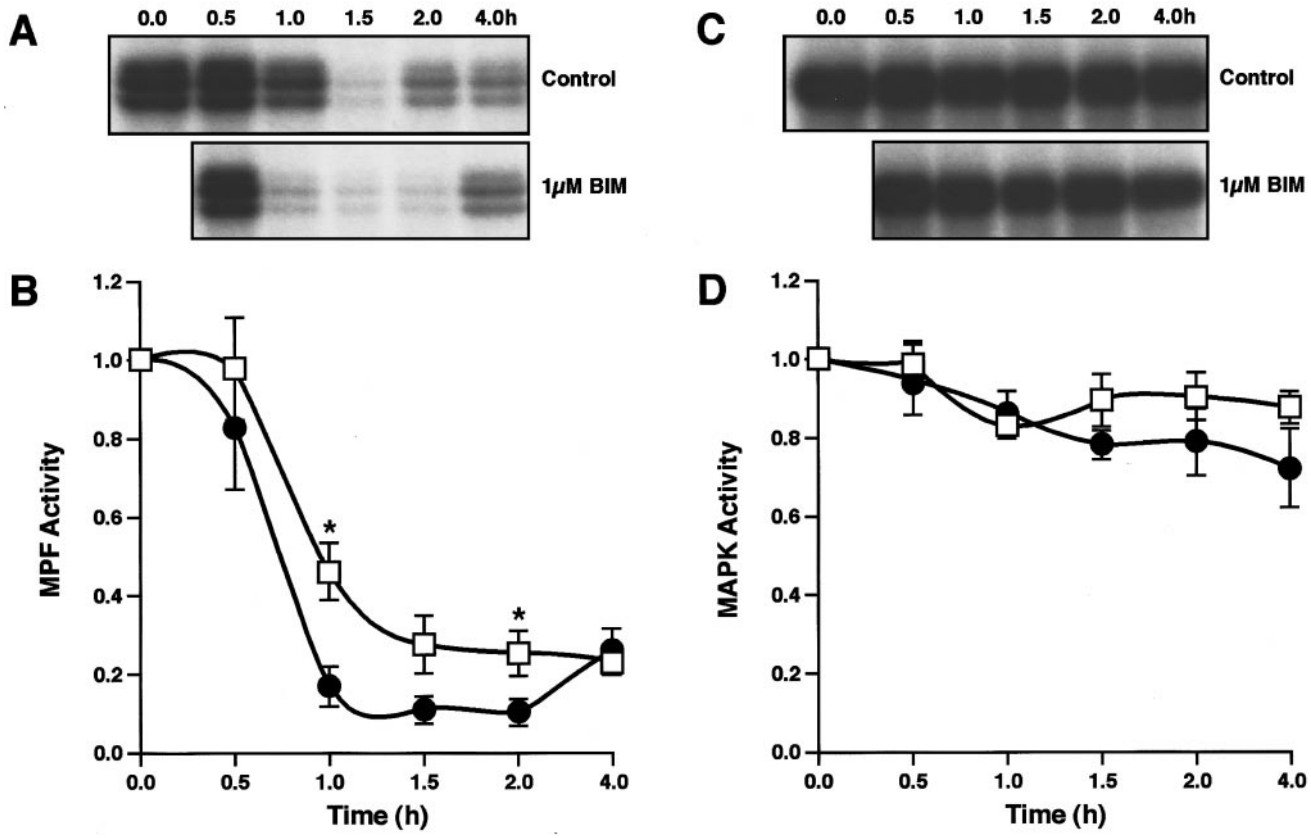

FIG. 3. Treatment with the PKC inhibitor BIM lowers MPF, but not MAPK, activity. Both MPF (A, B) and MAPK (C, D) activities were evaluated in LTXBO oocytes during the MI-to-MII transition, in response to 1.0 μM BIM. COC were collected and placed in culture for 14 h. At the end of culture, the cumulus cells were removed, and denuded late MI-stage oocytes cultured in MEM (□) or MEM supplemented with 1.0 μM BIM (●). Oocytes were collected at 30-min intervals for the next 2 h, and at 4 h post treatment. Representative gels are shown for both MPF (A) and MAPK (C) activities measured in same reaction using a total of 10 denuded oocytes. Densitometry analysis was performed and all data are expressed as a percent (mean ± SEM) of the value at 14 h for MPF (B) and MAPK (D). *, $P < 0.05$ between the control and BIM-treated group.

Statistical Analysis

All data are presented as mean percentages (±SEM) of a minimum of four independent experimental replicates. For evaluation of the differences between groups, all percentages were subjected to arcsin transformation. The transformed data were then analyzed by ANOVA and the means compared using a Fisher's protected least-significant difference posthoc test using Statview for Macintosh (Abacus Concepts, Berkeley, CA). Significance was assigned at $P < 0.05$.

RESULTS

Protein Kinase C Activity Is Elevated in Late MI-Stage LTXBO Oocytes

Phosphorylation of the PKC substrate, MARCKS psd peptide, was used to measure PKC activity in LTXBO oocytes during meiotic maturation. PKC activity was detected in oocytes at the GV-stage and tended to increase with meiotic maturation after 4 and 8 h of cumulus-enclosed culture. The highest level ($P < 0.05$) of PKC activity was observed in late MI-stage oocytes collected at the end of a 14-h culture period (Fig. 1). Treatment of denuded late MI-stage oocytes with the specific PKC inhibitor BIM (1.0 μM), for 4 h in culture, effectively lowered ($P < 0.05$) endogenous PKC activity (Fig. 1). No PKC activity was detected in GV-stage oocyte control samples when 1.0 μM BIM was added to the reaction buffer, or the MARCKS substrate was omitted (data not shown).

Suppression of Endogenous PKC Activity Promotes a Decrease in MPF Activity and Entry into Anaphase I

Since high levels of PKC activity were detected in late MI-stage oocytes, an assessment was undertaken to deter-

mine whether suppression of PKC activity at this time would influence subsequent meiotic maturation. The 2-h interval, between 14 and 16 h after *in vitro* maturation, was identified as the critical time during which LTXBO oocytes begin to undergo a delayed MI-to-MII transition. Treatment with 1.0 μM BIM to lower PKC activity in late MI-stage oocytes increased ($P < 0.05$) the percentage of oocytes that entered anaphase I and progressed to MII (Fig. 2). Within 1 h of BIM treatment, approximately 60% of the oocytes had extruded a first polar body compared with 30% in the control group. By 2 h, 90% of the BIM-treated oocytes had progressed to MII, relative to 70% in the control group (Fig. 2). The progression of chromosome segregation was confirmed with propidium iodide staining (data not shown). DNA was evident in the polar body of all oocytes, both control and BIM-treated, which progressed to MII and thus confirmed the segregation of homologous chromosomes.

Both MPF and MAPK activity were also evaluated during the MI-to-MII transition, in response to BIM treatment. When late MI-stage oocytes were treated with 1.0 μM BIM, a decrease ($P < 0.05$) in MPF activity was observed compared to the control group (Fig. 3A and B). MPF activity was lower ($P < 0.05$) in the BIM-treated oocytes within 1 h of exposure, and remained lower for the next hour. MPF activity began to increase in the BIM-treated oocytes within 4 h, even with continued exposure to the PKC inhibitor. In contrast to MPF, MAPK activity was unaffected by treatment with the PKC inhibitor BIM (Fig. 3C and D), and remained elevated during the MI-to-MII transition in both the control and BIM-treated group. Taken together, these results demonstrate that suppression of endogenous PKC activity, in LTXBO oocytes, transiently lowers MPF activity and promotes entry into anaphase I without affecting MAPK activity.

PKC-δ Localizes to the Meiotic Spindle at the Anaphase I-to-Telophase Transition

Although protein expression of various PKC isoforms (α, β, δ, λ, and ζ) has been detected in mouse oocytes, only mRNA transcripts of PKC-δ and -λ are detected (Gangeswaran and Jones, 1997; Pauken and Capco, 2000; Downs *et al.*, 2001). We have confirmed the same mRNA expression profile in LTXBO oocytes and detected higher protein expression of PKC-δ relative to PKC-λ (data not shown). Moreover, the "atypical" PKC-λ is not responsive to phorbol ester stimulation (Mellor and Parker, 1998). We, therefore, focused on assessing the subcellular expression pattern of PKC-δ in LTXBO oocytes during meiotic maturation. Oocytes arrested at prophase I with an intact GV exhibit diffuse cytoplasmic staining for PKC-δ (data not shown). Cytoplasmic staining was also evident in oocytes at the late MI-stage collected after a 14-h culture period; at this stage, PKC-δ also appears associated with the meiotic spindle (Fig. 4A). As oocytes progressed from MI to MII, PKC-δ was clearly and consistently colocalized with the spindle throughout the anaphase I (Fig. 4B)-to-telophase transition (Fig. 4C). PKC-δ was then observed in association with the chromosomes in oocytes at metaphase of meiosis II (Fig. 4D).

Modulation of PKC Activity Influences Spontaneous Parthenogenetic Activation

Previous studies have demonstrated an association between a prolonged MI-stage and spontaneous parthenogenetic activation by LT oocytes (Eppig *et al.*, 1996). We therefore assessed whether LTXBO oocyte activation and entry into interphase were influenced by PKC activity. Late MI-stage oocytes were collected after a 14-h cumulus-enclosed culture, denuded, and treated with increasing concentrations of either a PKC inhibitor (BIM) or a PKC agonist (PMA). Emission of the first polar body and PN formation were used as indicators of progression to MII and spontaneous activation, respectively. DNA staining with propidium iodide clearly illustrates tightly condensed chromosomes in oocytes that are arrested at MII (Fig. 5A). In contrast, highly decondensed chromatin was evident in oocytes that formed a PN and entered interphase after the first meiotic division (Fig. 5B). Note that decondensed chromatin is also present in the polar body. Treatment of denuded late MI-stage oocytes with increasing concentrations of BIM reduced ($P < 0.05$) the incidence of PN formation by LTXBO oocytes in a dose-dependent manner (Fig. 5D). Oocytes treated with BIM extruded the first polar body and remained arrested at MII (Fig. 5C); hence, subsequent entry into the first mitotic cycle and cleavage to the two-cell stage was reduced ($P < 0.05$) (Fig. 5E). In contrast, treatment with PMA (a PKC agonist) promoted a higher ($P < 0.05$) incidence of PN formation (Fig. 6A) and development to the two-cell stage by LTXBO oocytes (Fig. 6B). Thus, modulation of PKC activity in late MI-stage LTXBO oocytes can influence subsequent spontaneous activation, with a decrease in PKC activity effectively lowering oocyte activation rates.

To determine when oocytes were most susceptible to modulation of PKC activity, late MI-stage oocytes were collected after a 14-h cumulus-enclosed culture, denuded, and treated with 1.0 μM BIM either immediately (14 h) or at subsequent 2-h intervals until 20 h (16, 18, and 20 h) (Fig. 7). The incidence of PN formation was dramatically reduced ($P < 0.05$) in oocytes exposed to BIM at 14 h. Treatment at 16 h also lowered PN formation ($P < 0.05$), but to a lesser extent than that observed with exposure at 14 h. And, no reduction in PN formation was observed when the oocytes were treated with BIM at, or after, 18 h (Fig. 7A). The same pattern was noted in development to the 2-cell stage (Fig. 7B). The 14- to 16-h period after *in vitro* maturation has been identified as the critical interval when LTXBO oocytes begin to enter anaphase I. These results, therefore, demonstrate that entry into interphase is prevented only when endogenous PKC activity is suppressed before, or during, the MI to MII transition. This suggests that the defect(s), which promote activation in LTXBO oocytes are directly

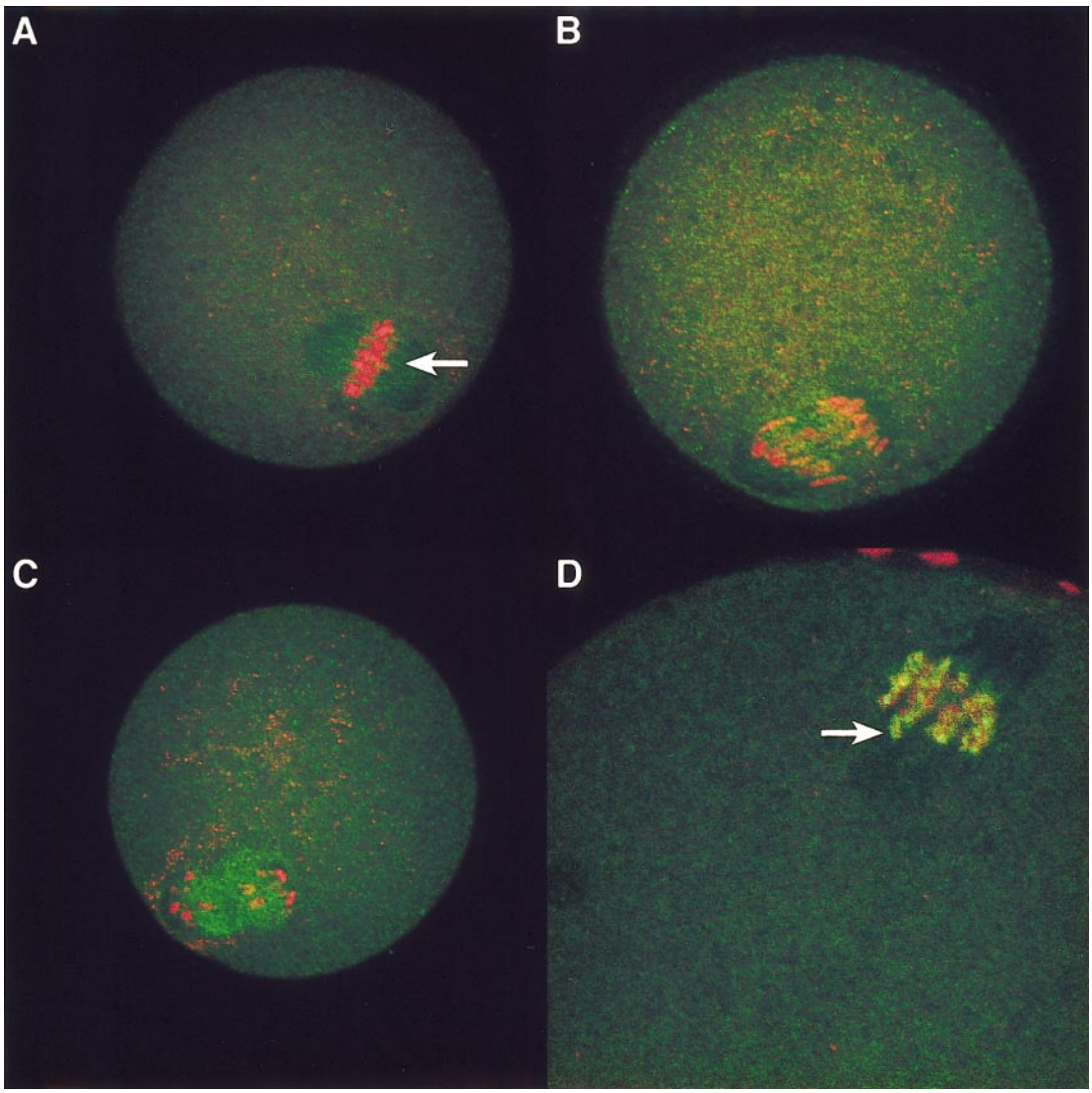

FIG. 4. PKC-δ localizes to the meiotic spindle during the anaphase I to telophase transition and is associated with the chromosomes at MII. Confocal microscopy analysis of LTXBO oocytes during the MI-to-MII transition demonstrated that some PKC-δ begins to localize with the meiotic spindle at MI, as indicated by the arrow (A). During anaphase I (B) and the late anaphase-to-telophase transition (C), PKC-δ was consistently localized with meiotic spindle and eventually with the chromosomes (arrow) at MII (D). PKC-δ is shown in green and the chromosomes in red.

associated with the late triggering of anaphase I, and whether an oocyte will arrest at MII or prematurely exit meiosis is determined at the late anaphase I to telophase transition. PN formation and chromatin decondensation in the activated eggs become evident several hours after this critical transition point.

In a final experiment, it was determined whether treatment with the PKC inhibitor BIM influences spontaneous activation rates in oocytes from LTXBO mice bearing a null allele for *Mos*. MOS is an important upstream regulator of the MAPK cascade and oocytes from Mos^{tm1Ev}/Mos^{tm1Ev} mice lack sufficient MAPK activity to sustain a stable arrest at MII and undergo spontaneous activation (Colledge *et al.*, 1994; Verlhac *et al.*, 1996). High levels of PKC downregulate MAPK activity in mouse oocytes at MII (Sun *et al.*, 1999). Hence, the ability of PKC to influence activation in LTXBO oocytes in the absence of MOS (and MAPK activity) was assessed. Late MI-stage oocytes were collected from both $Mos^{tm1Ev}/^{+}$ and homozygous Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice and treated with 1.0 μM BIM, as previously described. Assessment of PN formation rates demonstrated a significant decrease in spontaneous activation by $Mos^{tm1/Ev}/^{+}$

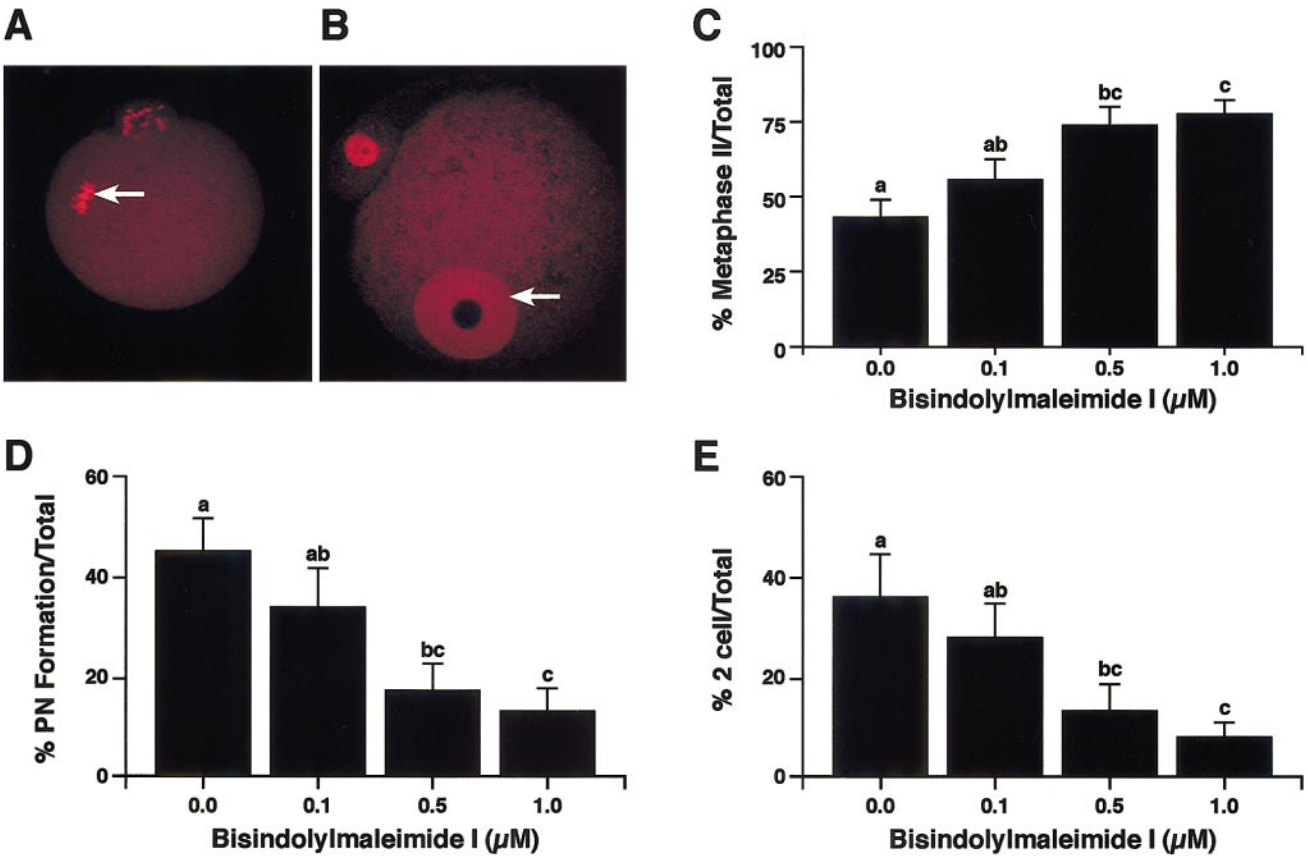

FIG. 5. Treatment with the PKC inhibitor BIM lowers spontaneous activation by LTXBO oocytes. Chromatin configuration of LTXBO oocytes were evaluated with propidium iodide staining (shown in bright red) to determine arrest at MII (A) as evidenced by the maintenance of condensed chromosomes (arrow), or entry into interphase by spontaneously activated oocytes (B) that are characterized by highly decondensed chromatin (arrow) and PN formation. The incidence (mean ± SEM) of progression to MII (C), PN formation (D), and development to the two-cell stage (E) were evaluated in response to increasing concentrations (0, 0.1, 0.5, and 1.0 μM) of BIM. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

LTXBO oocytes treated with BIM, relative to the control group cultured in MEM alone (Fig. 8). However, treatment with BIM failed to prevent spontaneous activation by oocytes collected from Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice (Fig. 8). These results suggest that the mechanism of activation in oocytes from the LTXBO strain can be distinguished from that of oocytes from *Mos*-null mice, and that the initial defect(s) in LTXBO oocytes that trigger activation occur upstream of the MAPK cascade.

DISCUSSION

Oocytes of the LTXBO mouse strain express specific defects in the regulatory mechanisms that govern the first meiotic division. These oocytes exhibit a delayed entry into anaphase I that is often followed by an inappropriate entry into interphase after the first meiotic division (Eppig *et al.*, 1996; Ciemerych and Kubiak, 1998; Hirao and Eppig, 1999). In the current study, we demonstrate that both anaphase I triggering and spontaneous activation by LTXBO oocytes are regulated by PKC. Endogenous PKC activity was highest in late MI-stage oocytes and correlated with the localization of PKC-δ at the meiotic spindle. Suppression of PKC activity at the late MI-stage promoted a transient decrease in MPF activity and entry into anaphase I. Inhibition of PKC activity also prevented the transition into interphase, but only when PKC activity was reduced in oocytes before the progression to MII. Taken together these results demonstrate that PKC participates in the regulatory mechanisms that delay entry into anaphase I in LTXBO oocytes. Moreover, the inappropriate entry into interphase observed in these oocytes is directly associated with the delayed triggering of anaphase I. This suggests that loss of regulatory control over PKC during the first meiotic division can disrupt the MI-to-

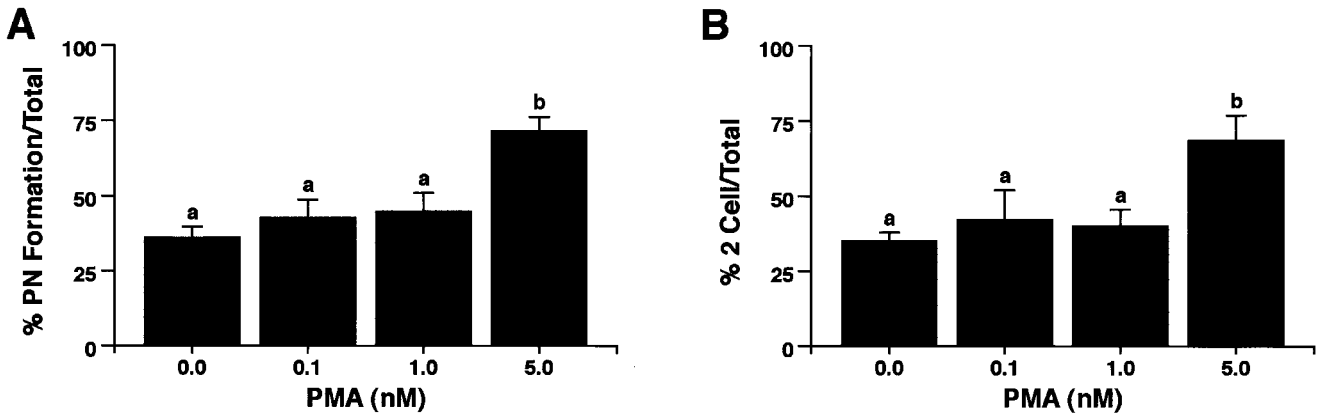

FIG. 6. Treatment with the phorbol ester PMA, a PKC agonist, promotes spontaneous activation by LTXBO oocytes. The incidence (mean ± SEM) of PN formation (A) and development to the two-cell stage (B) by LTXBO oocytes were evaluated in response to increasing concentrations (0, 0.1, 1.0, and 5.0 nM) of PMA, at 24 and 48 h after initial COC recovery, respectively. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

MII transition and thus promotes precocious exit from meiosis and the transition into the first mitosis.

PKC Activity Is Associated with Entry into Anaphase I

Endogenous PKC activity was detected in LTXBO oocytes at prophase I; this activity increased with meiotic maturation and was highest at the late MI-stage. Suppression of PKC activity at this stage transiently lowered MPF activity and promoted entry into anaphase I by late MI-stage oocytes. These data suggest that PKC may extend the MI-stage in LTXBO oocytes by sustaining MPF activity and thereby delay the entry into anaphase I. The mechanism(s) through which PKC maintains MPF activity is not known. Studies with somatic cells demonstrate that PKC interacts with the regulatory factors that influence MPF activity. For example, PKC suppresses MPF activation through the down-regulation of the phosphatase cdc25 (thus inhibiting cdc2 dephosphorylation), or through the induction of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, which blocks cdc2 activity (Livneh and Fishman, 1997; Black, 2000). Our

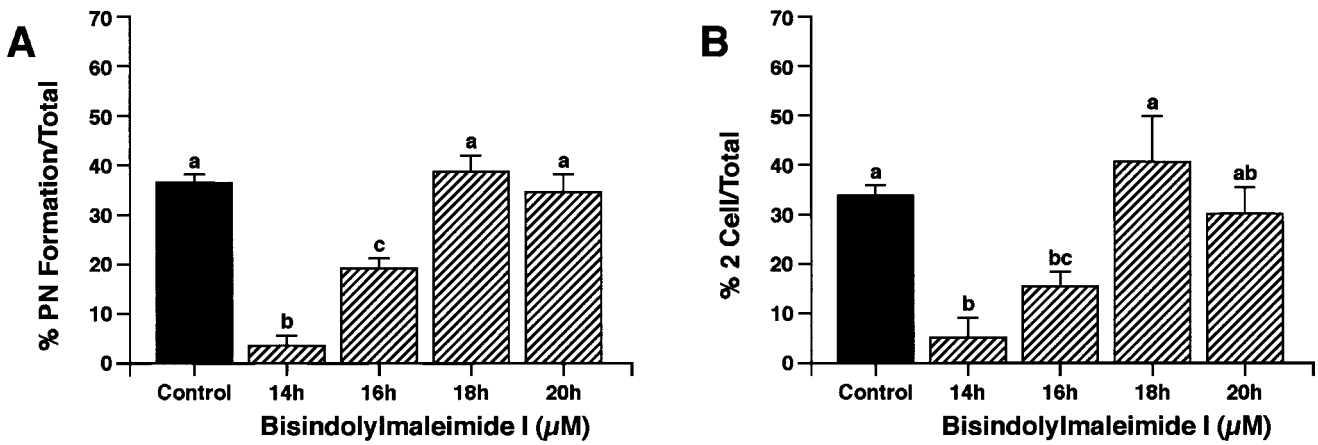

FIG. 7. To prevent spontaneous activation, endogenous PKC activity must be reduced in oocytes before the progression to MII. COC were collected and placed in culture for 14 h. At the end of culture, the cumulus cells were removed and late MI-stage oocytes were cultured in MEM (solid bars) or MEM supplemented with 1.0 μM BIM (hatched bars). The oocytes were treated with BIM either immediately (14 h) or at subsequent 2-h intervals until 20 h (at 16, 18, and 20 h after initial COC recovery). The incidence (mean ± SEM) of PN formation (A) and development to the two-cell stage (B) were evaluated at 24 and 42 h after initial COC recovery, respectively. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

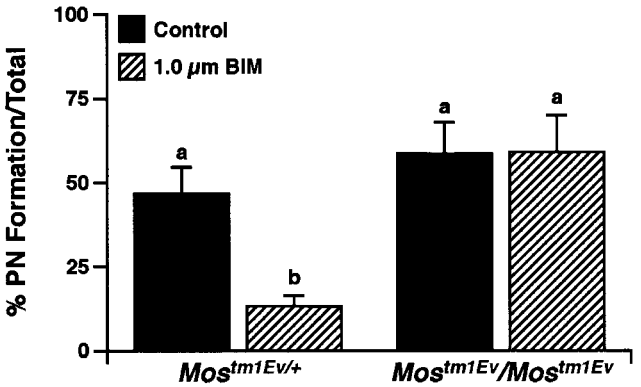

FIG. 8. Treatment with the PKC inhibitor BIM lowers the incidence of spontaneous activation by oocytes from $Mos^{tm1Ev}/^{+}$, but not homozygous Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice. COC were collected from $Mos^{tm1Ev}/^{+}$ and Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice and placed in culture for 14 h. At the end of culture, the cumulus cells were removed and denuded late MI-stage oocytes were cultured in MEM (solid bars) or MEM supplemented with 1.0 μM BIM (hatched bars). The incidence (mean $\pm$ SEM) of PN formation was determined after a 10-h incubation. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

current results suggest that PKC may also regulate MPF activity during meiosis I. Previous investigations with MII-arrested eggs, that normally exhibit high MPF activity, report variable responses to PKC. One study showed that treatment with PKC agonists triggers MPF inactivation and promotes mouse egg activation (Colona *et al.*, 1997). Yet, in other studies, no significant decrease in MPF activity was detected in response to PKC activation (Moore *et al.*, 1995). In *Xenopus* eggs, PKC stimulation reduces MPF activity, but only when calcium levels are high (Bement and Capco, 1991). These conflicting results have previously been attributed to species and/or strain differences along with the degree of PKC stimulation. However, it is also important to consider that the response to PKC may very well depend on the stage of the cell cycle. In the current study, oocytes were assessed at the late MI-stage. Although MPF activity is high during both MI and MII these divisions differ sharply in their final outcome and, thus, the mechanisms that regulate MPF activity during these two stages might also differ. Studies with *Mos*-null mice demonstrate that the regulatory factors necessary to sustain MI and MII arrest are not the same. While MOS is an important component of CSF and is critical for the maintenance of a stable MII arrest, it is not essential during MI (Choi *et al.*, 1991; Colledge *et al.*, 1994). Studies with *Mos*-null LTXBO mice also suggest that MOS does not instigate MI arrest in LT oocytes (Hirao and Eppig, 1997). Rather it has been proposed that a delay in acquisition of competence to enter anaphase I may instigate a prolonged MI-stage by allowing CSF activity to rise and maintain MPF activity (Hirao and Eppig, 1999). CSF activity does in fact develop in LT oocytes during MI (Ciemerych and Kubiak, 1998). However, the factors involved in the regulation of CSF activity during MI remain poorly understood. Our current findings suggest that since PKC can delay the exit from MI it might play a role in the regulation of CSF activity during meiosis I.

Earlier work from this laboratory has demonstrated that MPF activity is maintained in late MI-stage LT oocytes, in part, by restricted degradation of cyclin B (Hampl and Eppig, 1995). Given that suppression of PKC activity reduces MPF activity, it will be important to ascertain if PKC affects the stability of cyclin B levels. The relatively rapid response to lower PKC activity suggests a possible effect on cyclin B degradation, rather than synthesis, rates. For example, PKC might interact with the ubiquitin-targeting mechanism that is ultimately responsible for cyclin B degradation. Alternatively, since cyclin B degradation requires an intact spindle (Kubiak *et al.*, 1993), PKC might influence the integrity of the meiotic spindle. PKC agonists can induce the disassembly of spindle microtubules in mouse oocytes at MII (Moore *et al.*, 1995). The MI spindle is apparently assembled normally in LT oocytes (Ciemerych and Kubiak, 1998), but is elongated by the late MI-stage (Albertini and Eppig, 1995). Thus, inappropriately regulated PKC activity might mediate subtle, yet functional, disruptions in the MI spindle that impair cyclin B degradation and thereby delay entry into anaphase I. Moreover, it is possible that PKC plays a role in mediating kinetochore associations, since kinetochore interactions with microtubules regulate exit from MI (Brunet *et al.*, 1999).

That PKC regulates MPF activity and entry into anaphase I was supported by our observations that at least one PKC family member, PKC-δ, is associated with the meiotic spindle during the MI to MII transition and eventually with the chromosomes at MII. The translocation of some PKC-δ to the meiotic spindle was coincident with the observed increase in PKC activity at the late MI-stage. These results suggest a role for this kinase in cell-cycle progression and indicate that other spindle-associated proteins, such as MPF, MAPK, or proteasomes that may facilitate timely degradation of cyclin B (Josefsberg *et al.*, 2000), are possible target substrates for PKC-δ. Although expression of PKC-δ has been demonstrated in oocytes from other mouse strains at both the GV and MII stage by Western blot analysis (Gangeswaran and Jones, 1997; Pauken and Capco, 2000; Downs *et al.*, 2001), it is possible that this kinase may be inappropriately regulated in LTXBO oocytes. Current studies are underway to evaluate the subcellular localization of PKC-δ during meiotic maturation in control strains, and to more specifically define the role of PKC-δ during meiotic maturation in both control and LT oocytes.

Modulation of PKC Activity Regulates Spontaneous Activation

In addition to promoting entry into anaphase I, suppression of PKC activity reduced spontaneous activation by LTXBO oocytes. Previous studies indicate that a delay at MI

is necessary, but insufficient, to promote spontaneous activation in LT oocytes (Maleszewski and Yanagimachi, 1995; Eppig *et al.*, 1996). In the current study, we extended these findings and demonstrated that critical disruptions in cell-cycle regulation in LTXBO oocytes, which promote entry into interphase, occur specifically during the anaphase I to telophase transition. Spontaneous activation was prevented only when PKC activity was inhibited before oocyte progression to MII, which indicates that activation of LTXBO oocytes is directly linked with the triggering of anaphase I.

The precise role of PKC in LTXBO oocyte activation remains to be determined. It is possible that elevated, or inappropriately regulated, PKC activity in late MI-stage oocytes directly promotes the transition into interphase. Previous investigations suggest that PKC agonists promote MII egg activation and entry into interphase (Bement and Capco, 1991; Colona *et al.*, 1997; Gallicano *et al.*, 1997a,b). Therefore, lowering endogenous levels of PKC activity in LTXBO oocytes may block activation by preventing the PKC-mediated entry into interphase. This would imply that PKC has a dual role in LT oocytes and can both delay entry into anaphase I and stimulate spontaneous activation. Alternatively, it is possible that PKC promotes oocyte activation only indirectly, as a consequence of delaying the entry into anaphase I. It is largely accepted that the predisposition of mouse oocytes to respond to an activating stimulus (fertilization or parthenogenetic agent) increases gradually after extrusion of the first polar body (Xu *et al.*, 1997). Hence, as MII eggs "age" they become more prone to enter interphase upon activation. MI-stage LT oocytes respond to ethanol activation considerably earlier after GVBD than control MII eggs (Ciemerych and Kubiak, 1998). This suggests that LT oocytes acquire a predisposition to enter interphase during a prolonged MI-stage, and that there might be a point at which these oocytes are no longer able to restabilize MFP activity when it decreases at anaphase. In effect entry into a late anaphase I would act as the "activating stimulus." In this scenario, lowering PKC activity would prevent activation by triggering entry into anaphase I before oocytes reach this critical point when they would be unable to restabilize MPF.

The specific mechanism(s) that initiate activation in LT oocytes are not known. Recent work suggests that MOS prevents a meiotic/mitotic transition after the first meiotic division until after fertilization (Tachibana *et al.*, 2000). However, previous studies (Hirao and Eppig, 1997; Ciemerych and Kubiak, 1998) and the current data demonstrate that LT oocytes are not deficient in MOS expression or MAPK activity, although a disruption in the MAPK cascade might occur. Moreover, PKC agonists down-regulate MAPK activity in mouse MII eggs (Sun *et al.*, 1999). To determine whether PKC regulates LTXBO oocyte activation in the absence of MOS and MAPK activity, late MI-stage oocytes from $Mos^{tm1Ev}/^{+}$ and Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice were treated with the PKC inhibitor BIM. Suppression of PKC activity significantly reduced the incidence of spontaneous activation by $Mos^{tm1Ev}/^{+}$ LTXBO oocytes, but failed to prevent activation by oocytes from homozygous Mos^{tm1Ev}/Mos^{tm1Ev} LTXBO mice. This contrasting response to PKC suppression indicates that the mechanism(s), which initiate activation in LTXBO oocytes differs from that of *Mos*-null mice. Moreover, as shown here, activation by LTXBO oocytes can be regulated by PKC and is associated with the delayed triggering of anaphase I. Taken together, these results indicate that the initial defect(s) in LTXBO oocytes, which promote eventual entry into interphase, occur upstream of the MAPK cascade. Nevertheless, MOS-regulated MAPK activity normally decreases after fertilization and parthenogenetic activation. Hence, a decrease in MAPK will presumably ensue in LTXBO oocytes as a consequence of the initial defect(s) that promote activation, but seems unlikely to be the primary lesion in these oocytes.

In summary, the results of the current study demonstrate that PKC regulates the progression of meiosis I in LTXBO oocytes. Defects in the progression of meiosis I in these oocytes delay entry into anaphase I and promote a premature entry into interphase after the first meiotic division. Our extended characterization of the meiotic defects in the LT model indicate that entry into interphase is initiated, with the delayed triggering of anaphase I, upstream of the MAPK cascade. Moreover, aberrant PKC activity and/or regulation participate in the expression of this observed phenotype. Suppression of endogenous PKC activity promoted a transient decrease in MPF activity and entry into anaphase I, thus suggesting that PKC might play a role in the regulation of CSF activity during meiosis I. Suppression of PKC activity during the anaphase I-to-telophase transition also promoted a stable arrest at MII and, thereby, prevented entry into interphase. Whether PKC plays a critical role in the normal progression of meiosis I remains to be determined. However, the current data with the LTXBO model provide clear evidence that loss of regulatory control over PKC activity disrupts the timing of the critical MI-to-MII transition, and this disruption promotes a premature exit from meiosis and the transition into mitosis.

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